Institut für Veterinärphysiologie
der Vetsuisse-Fakultät Universität Zürich

Direktor : Prof. Dr. med. vet. Max Gassmann

Arbeit unter Leitung von Prof. Dr.med. Mario Schiffer , Medizinische Hochschule Hannover, Hannover, Deutschland

Analyse des Glucose-Stoffwechsels in Podozyten unter diabetischen und nicht-diabetischen Bedingungen

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Nicole Stieger

Tierärztin
von Zürich ZH, Schweiz

genehmigt auf Antrag von

Prof. Dr. med. vet. Thomas Lutz, Referent

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Index

1.Zusammenfassung

Diabetische Nephropathie ist die häufigste Ursache für chronisches Nierenversagen in der westlichen Welt. Der Podozytenverlust im Glomerulum spielt eine wichtige Rolle in der Initialphase der diabetischen Glomerulopathie. Verschiedene Faktoren führen im diabetischen Milieu zur Hypertrophie, Ablösung und Apoptose des Podozyten. Mitochondriale Dysfunktion spielt in der diabetischen Nephropathie eine wichtige Rolle. In der vorliegenden Arbeit haben wir das metabolische Profil von Podozyten unter diabetischen Bedingungen untersucht. Dazu haben wir in murinen Podozyten den NADH-Gehalt, die Sauerstoffrate (OCR) und die einzelnen Atmungskettenkomplexaktivitäten gemessen. Die Zellen wurden entweder für 48h oder für mindestens 10 Passagen in Medium mit erhöhtem Glukosegehalt (30mM) kultiviert. Bei den unter chronisch hyperglykämischen Bedingungen kultivierten Podozyten war im Vergleich zu der osmotischen Kontrolle die basale OCR erhöht, ebenso die OCR nach der Oligomycin-, FCCP- und Rotenon-Injektion. Nach der Stimulation mit TGF-β (5ng/ml) für 24h zeigten Podozyten trotz einer reduzierten Citratsynthase-Aktivität ähnliche Veränderungen in der OCR. In allen untersuchten Bedingungen war der NADH-Gehalt in den Zellen erhöht und die Atmungskomplexaktivitäten verändert. Dadurch konnten wir erstmals zeigen, dass zwei wichtige Faktoren

im Diabetes den Metabolismus und die Mitochondrienfunktion von Podozyten maßgeblich beeinflussen.

2. Summary

Diabetic nephropathy is the most common cause of chronic renal failure in the industrialized countries. Depletion of podocytes plays an important role in progression of diabetic glomerulopathy. Various factors in the diabetic milieu lead to serious podocyte stress driving the cells towards cell cycle arrest, hypertrophy, detachment and apoptosis. Recent studies indicate that mitochondrial dysfunction is a key factor in diabetic nephropathy. In the present study we investigated metabolic profiles of podocytes under diabetic conditions. We examined NADH content, oxygen consumption rates (OCRs) and oxidative phosphorylation (OXPHOS) complex activities in murine podocytes. Cells were either exposed to high glucose for 48h, cultured for 10 passages under high glucose conditions (30mM), or incubated with TGF-β (5ng/ml) for 24h. We found in cells prolonged exposed to high glucose a significantly increased OCR at baseline and after injection of oligomycin indicating a changed respiration. Podocytes stimulated with a pro-apoptotic concentration of TGF-β displayed similar bioenergetics profiles, even with

decreased citrate synthase activity. In all tested conditions we found a higher cellular NADH content as well as changes in activities of respiratory chain complexes. In summary, we provide for the first time evidence that key factors of the diabetic milieu induce changes in glucose metabolism and mitochondrial function in podocytes.

3. Introduction

One of the major problems in diabetes is decreased cellular glucose uptake leading to hyperglycemia which affects various organs including the kidney. Podocytes as a part of the glomerular filtration barrier play an important role in the development of diabetic nephropathy. As an early event in diabetic glomerulopathy, loss of podocytes is detectable due to dedifferentiation, detachment and finally apoptosis.[1,2,3] Excessive amounts of glucose disturb cellular metabolism and induce mitochondrial dysfunction. In this context, several groups postulated various pathways in mesangial cell and proximal tubule cell metabolism which are affected by hyperglycemia. Enhanced flux rate through the polyol pathway and an increased activity of the hexamine pathway which lead to enhanced levels of NADH were

documented, however in podocytes these pathways have not been investigated[4,5,6]. Mitochondria are crucial to maintain energy homeostasis and gluconeogenesis. They also play a central role in late apoptosis and are a major source of reactive oxygen species (ROS) which may induce apoptosis. In living cells, NADH-dehydrogenase (complex I) and Ubiquinon cytochrome c oxido reductase (complex III) are responsible for ROS production [7,8]. Thus far, mitochondrial function in podocytes has not been studied systematically. *Abe and coworkers* characterized the bioenergetic profiles of mouse podocytes and found a strong dependency on oxidative phosphorylation in the cells whereas glycolysis was negligible [9]. In the present study, we wanted to elucidate the bioenergetic profiles of podocytes under diabetic conditions to analyze how podocytes respond to elevated amounts of glucose and TGF-β regarding their metabolism, especially mitochondrial activity. For this purpose, we used the Seahorse Bioscience XF24 Extracellular Flux Analyzer to measure oxygen consumption rates (OCRs) and extracellular acidification rates (ECARs) in podocytes stimulated either with high glucose (30mM) or TGF-β (5ng/ml). Moreover, we characterized cell size and mitochondrial content of these cells, and analyzed the respiratory chain complex activities to reveal functional changes in mitochondria.

4. Material and Methods

4.1. Cell culture

Conditionally immortalized mouse podocytes were cultured as described previously [10]. The cells were grown in RPMI 1640 medium (Biochrom AG,

Berlin, Germany) containing 10% fetal calf serum (PAA, Pasching, Austria), 0.5 U/l penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 10 U/l Interferon-γ (Cell Sciences, Canton, MA) under permissive conditions at 33 °C and 5% CO_2. Podocytes used for the prolonged high glucose experiments were cultured in RPMI medium with a glucose concentration of 30mM or 30mM mannitol for 10 passages as osmotic control. For differentiation cells were transferred to nonpermissive conditions at 37 °C without Interferon-γ. Medium was changed every other day. After 7-10 days of differentiation podocytes were harvested

4.2. Alamar blue assay

Fluorimetrical analysis of resazurin reduction provides information about the metabolic activity, viability and proliferation of living cells which irreversibly convert the blue dye into the fluorimetrical measurable pink resofurin. In this study, we used the alamar blue assay (Sigma Aldrich, St. Louis, MO., USA) for determination of NADH levels in podocytes stimulated with various substrates. Cells were trypsinized after 7 days of differentiation and transferred into 96 well-plates at a density of 5 $x10^4$ cells / well. After 24h cells were stimulated with 30mM glucose/30mM mannitol for 48h or with TGF-β (5ng/ml) (R&D Systems, Minneapolis, MN) for 24h, respectively. Then the medium was changed and the alamar blue working solution (10% of final volume) was added. Fluorescence measurements were performed immediately at a wavelength of 560/590 nm with the Multi-Mode Microtiter Reader Synergy TM 4 and Gen5 TM software (BioTek Instruments, Winooski,

VT, USA) and replicated after 1h, 2h and 3h. Background fluorescence was subtracted and the results calculated in relative fluorescence units.

4.3. Measurement of oxygen consumption rates (OCR)

Oxygen consumption (OCR) and extracellular acidification rate (ECAR) were measured with the Seahorse Bioscience XF24 Extracellular Flux analyzer as described previously [11]. Differentiated podocytes were transferred into XF24-well cell culture microplates (Seahorse Bioscience, North Billerica, MA, USA) at a density of 6.0 x10^4 cells per well and incubated 24 h at 37°C and 5% CO_2 prior to stimulation. After incubation with 30mM glucose or 30mM mannitol for 48h or 5ng/ml TGF-β for 24h the medium was replaced with 675 µl assay medium (Dulbecco's modified Eagle Medium with 5mM glucose, pH 7,4; Sigma Aldrich, St. Louis, MO, USA) and adjusted for 1h in a CO_2-free incubator at 37°C. Following a period of basal OCR and ECAR measurements oligomycin (0.25µM), FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 0.25 µM) and rotenone (0.1µM) were added at defined time points. Results were calculated as an average of 3 (basal rate 4) points before and after the corresponding injection. All substances were purchased from Sigma-Aldrich (St. Louis., MO, USA).

4.4. Measurement of cell size

For all size measurements normal podocytes and podocytes for 10 passages on 30 mM glucose/mannitol were differentiated on coverslips. Normal cells were stimulated with 30mM glucose/30mM mannitol for 48h. After staining with the endocytosis marker Cholera toxin subunit B (Alexa Fluor 488

conjugated) (Invitrogen, Carlsbad, CA, USA) at 4°C for 1h, cells were washed three times with PBS and fixed immediately with 4% paraformaldehyde. Pictures were taken on a LEICA DMLB microscope (Leica, Bonn, Germany). The cells were analyzed by measuring the median diameter with ImageJ software (National Institutes of Health, USA). The results were calculated in relative units.

4.5. Fluorimetrical analysis and immunocytochemistry of mitochondrial marker

Differentiated normal podocytes or podocytes exposed to 30mM glucose/30mM mannitol for 10 passages were seeded into 96-well plates at a density of 2.0 x 10^4 cells per well. Podocytes were incubated with 30mM glucose/30mM mannitol for 48h or used directly in the experiments. After incubation with Mitotracker Orange ™ (100nM, Invitrogen, Carlsbad, CA, USA) at 37° C and 5% CO_2 for 45 minutes cells were washed two times with medium. Fluorescence measurements were performed immediately at a wavelength of 544/576 nm with the Multi-Mode Microtiter Reader Synergy TM 4 and Gen5 TM software (BioTek Instruments, Winooski, VT, USA). Background fluorescence was subtracted, and the results were calculated in relative fluorescence units. For immunocytochemistry, podocytes were differentiated on coverslips and fixed with 4 % paraformaldehyde after staining. Pictures were taken with the LEICA DMLB microscope.

4.6. Measurement of oxidative phosphorylation enzymes (OXPHOS)

Activities of respiratory chain complexes and citrate synthase as a mitochondrial marker were measured spectrophotometrically as described

previously [12] after the assays were adapted for podocytes. In brief, after differentiation of podocytes on plates containing 150 µg protein (determined by OD 600 nm) and two washing steps with HEPES buffer the cells were incubated with 50mM glucose for 15 min and broken by sonification 2x 10 s to expose mitochondrial complexes. Assays were run at 37 °C. All experiments were performed on an Agilent 8453 UV-Visible spectrophotometer using Microsoft Windows based Agilent ChemStation software (Agilent Technologies, Santa Clara, CA). The results were normalized to the total amount of protein which was measured according to Bensadoun and Weinstein [13].

4.6.1. Activity of complex I + III

After addition of cytochrome c (100mM) as an electron acceptor, the decrease of NADH was followed spectrophotometrically at 340 nm. The reaction was inhibited with rotenone [14].

4.6.2. Activity of Succinat-dehydrogenase and ubiquinone cytochrome c reductase (complex II +III)

In addition to KH_2PO_4 (50mM, pH 7), NaN_3 (250mM) and cytochrome c (100 µM) to activate the complexes, the assay buffer contained rotenone which inhibited complex I. The increase in reduced cytochrome c was followed at a wavelength of 550nm by adding succinate (1M). The reaction was inhibited with antimycin [15].

4.6.3. Activity of cytochrome c oxidase (complex IV)

For measurement of cytochrome c oxidase activity, the half-life of the oxidation of reduced cytochrome c was determined at 550 nm. When the required time was reached, the reaction was inhibited by potassium ferro cyanide [16].

4.6.4. Activity of ATPase (complex V)

ATPase activity was spectrophotometrically measured by a coupled assay using lactate dehydrogenase and pyruvate kinase in the direction of ATP hydrolysis. NADH consumption was followed at 340nm, oligomycin was added to inhibit the mitochondrial ATPsynthase [17,18]

4.6.5. Activity of citrate synthase

The reaction of oxalate and acetyl-CoA to citrate acid was assayed spectrophotometrically at a wavelength of 232 nm by measuring the decrease of acetyl-CoA. according to Srere [19].

4.7. Protein assay

Total amount of protein was determined photometrical according to Bensadoun and Weinstein [13,19]. A BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) was used.

4.8. Statistical analysis

All results are given as means ± SD. Data were analyzed using Excel statistical software and were compared by *Student's t-test*.

5. Results

5.1. Oxygen consumption rate

Oxygen consumption rate is changed by glucose and TGF-β in podocytes. To analyze podocyte metabolic activity after high glucose and TGF-β stimulation we determined the cellular respiration rates. To accomplish this we determined basal respiration rates in differentiated mouse podocytes, expressed as oxygen consumption rates (OCR) or as extracellular acidification rates (ECAR) with the Seahorse flux analyzer. Basal OCR was 62 ± 9 pmol/min (measured in initially 20.000 cells). After addition of oligomycin, an ATPase inhibitor, OCR decreased to 22 ± 8 pmol/min. The injection of the uncoupler FCCP increased the OCR to 40 ± 8 pmol/min whereas the following addition of rotenone, an inhibitor of NADH-dehydrogenase or complex I, diminished the OCR to a level of 22 ± 5 pmol/min (Figure 1A). We detected no differences in OCR between podocytes stimulated for 48h with 30mM glucose or 30mM mannitol, respectively (Figure 1B). However, when podocytes were exposed chronically to high glucose for 10 passages, we observed a significant increase in basal OCR in podocytes cultured with 30mM glucose (129 ± 12 % of high mannitol control, $p<0.001$). Furthermore, the cells showed a higher maximal respiratory capacity after FCCP injection than mannitol controls (150 ± 22 % of controls, $p<0.001$) (Figure 1C). A similar effect was detected in podocytes stimulated with 5ng/ml TGF-β for 24h (basal rate 146 ±18 % and 158 ± 21% of controls after FCCP-injection). In addition, OCR increased further when high glucose and TGF-β

stimulation were combined (data not shown). We did not find any differences in extracellular acidification rates (ECAR) under all investigated conditions (data not shown). For a more detailed characterization we analyzed oxidative phosphorylation enzymes in podocytes separately.

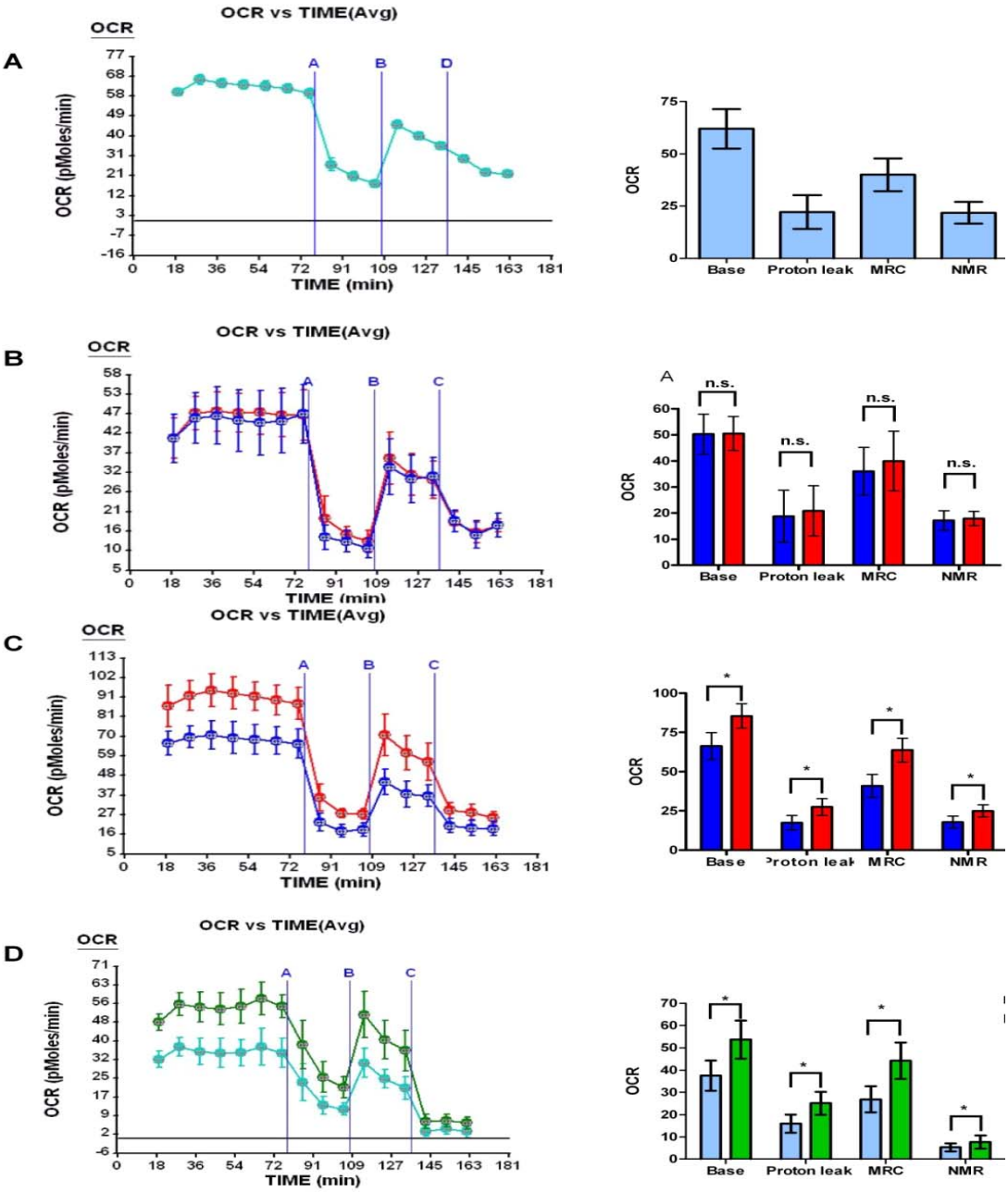

A
OCR vs TIME(Avg)
OCR
OCR (pMoles/min)
TIME (min)
A
B
D
Base
Proton leak
MRC
NMR
B
OCR vs TIME(Avg)
C
OCR vs TIME(Avg)
n.s.
D
OCR vs TIME(Avg)

Figure 1: High glucose and TGF-β cause changes in oxygen consumption in podocytes. Measurement of oxygen consumption rate (OCR) and respiratory capacity with various substrates of untreated podocytes (a) or podocytes stimulated with 30mM glucose / 30mM mannitol for 48h (b), for 10 passages (c), or with TGF-β (5ng/ml) for 24h (d). After the measurement of the baseline, oligomycin, FCCP and rotenone were added at defined time points. Scale bars represent the average of 4 (basal rate) or 3 points of OCR before and after the corresponding injection of two Independent experiments. The corresponding extracellular acidification rates (ECAR) for all conditions were not different (data not shown). (*p <0.05 compared by Student`s t- test; n.s. not significant). MCR maximal respiratory capacity, NMR non Mitochondrial respiration.

5.2. Activity of complex I+III

Glucose but not TGF- β causes changes in complex I+III activity. Stimulation of podocytes with high glucose for 48h significantly increased complex I+III activity (150 ± 31 % of the high mannitol controls, p=0.039 (Figure 2 A, panel b)). In contrast, we detected a significant decrease in podocytes cultured in high glucose for 10 passages (82 ± 6 of control, p=0.048 (Figure 2 A, panel c)). Stimulation with TGF-β had no effect on complex I+III activity (Figure 2 A, panel d).

5.3. Activity of complex II+III

Succinate dehydrogenase and ubiquinone cytochrome c reductase (complex II + III) activity was not affected by glucose and TGF-β. We found that the activity of complex II + III in podocytes cultured in 30mM glucose for 10 passages and podocytes stimulated with 5ng/ml TGF-β was slightly increased in comparison to the corresponding controls, whereas incubation with 30mM glucose for 48 h did not change the activity of the two complexes. However, the detected changes were not significant (Figure 2 B, panels b-d)

5.4. Activity of complex IV

Glucose and TGF-β led to alterations in cytochrome c oxidase activity (complex IV). The complex IV activity in podocytes stimulated with 30mM

glucose for 48h was significantly increased compared to the mannitol controls (205 ± 38 %, p=0.011 (Figure 2 C, panel b)). In contrast, in podocytes cultured with 30mM glucose for 10 passages we did not detect any differences in complex IV activity in comparison to the mannitol control (Figure 2 C, panel c). Stimulation with 5ng/ml TGF-β for 24h caused a significant decrease of cytochrome c oxidase activity (67 ± 7% of controls, p=0.041 (Figure 2 C, panel d)).

5.5. ATPase activity

ATPase activity was downregulated by glucose and TGF-β in podocytes. We found a reduction of ATPase activity in all tested conditions (Figure 2 D, panels b-d). However, the differences after treatment with TGF-β were not significant (64 ± 3 % vs. controls after 48h high glucose, p=0.012, 80 ± 15 % vs. controls after 10 passages in high glucose medium
p=0.038, 87 ± 4 % vs. controls, p=0.06 with TGF-β).

A=Komplex1,
B=Komplex2,C=Komplex4

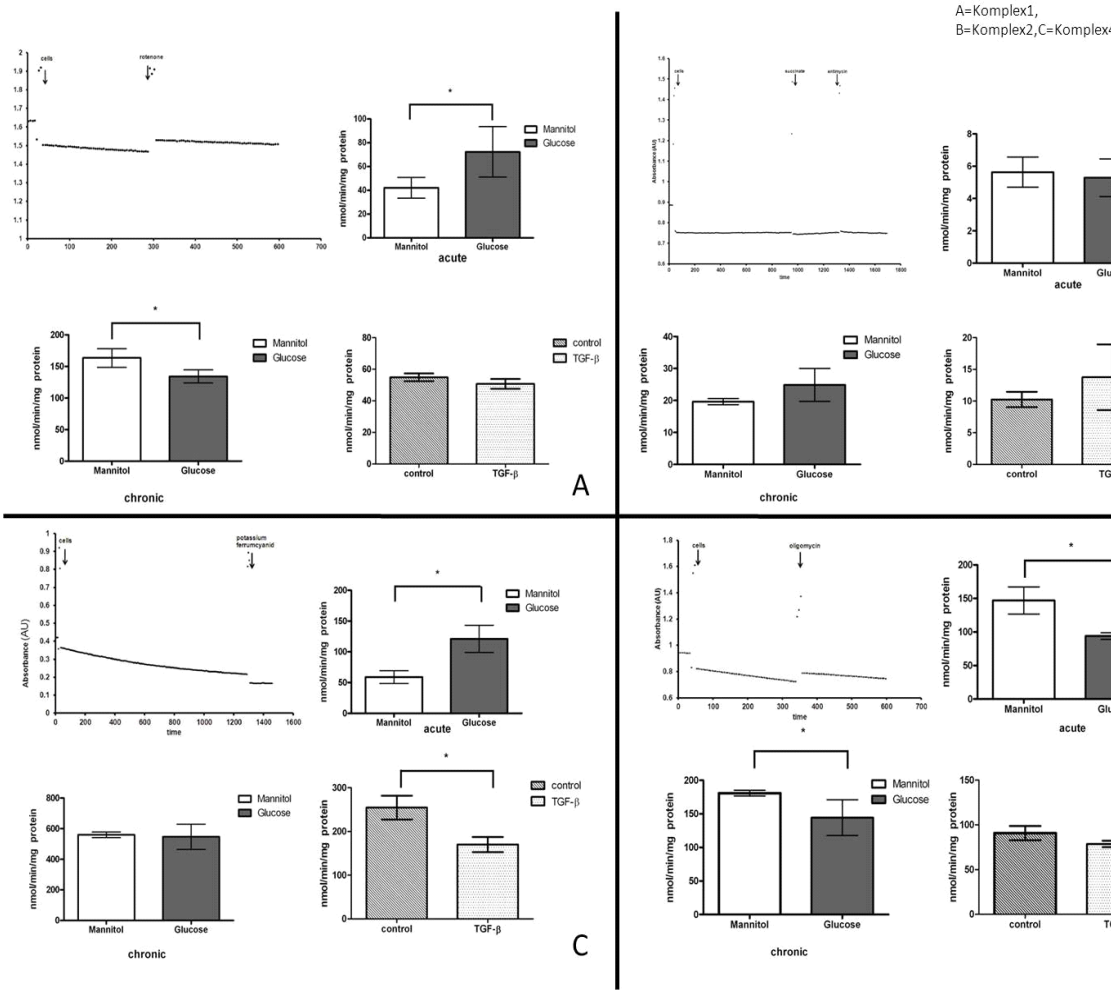
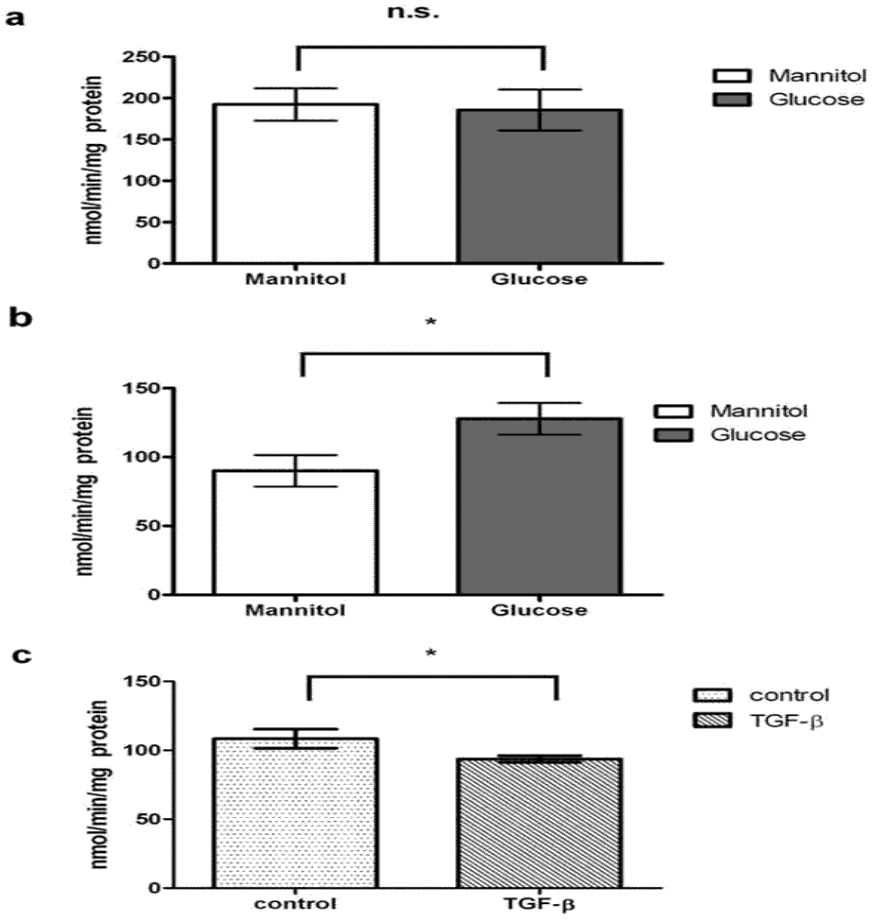


Figure 2: Effects of glucose and TGF-β on the respiratory chain complex Activity in podocytes. Changes in (A) activity of complex I + III, (B) Succinat dehydrogenase and ubiquinone cytochrome c-reductase (complex II +III), (C) activity of cytochrome-c oxidase (complex IV), (D) activity of ATP-ase (complex V) in podocytes. (a) represents an example of a measurement of the corresponding complex using spectrophotometry. (b) – (d) show changes of activities of respiratory complexes in podocytes after stimulation with 30mM glucose/30mM mannitol for 48h (b), for 10 passages (c) or with TGF-β (5ng/ml) for 24h (d). Each experiment was normalized to the total amount of protein. Results are expressed as means ± S.D. (n=3). (*p < 0.05 compared by Student's t test; n.s., not significant).

5.6 Activity of citrate synthase

High glucose and TGF-β modulate citrate synthase activity. Next we wanted to examine whether some of the measured differences could be explained by a different mitochondrial content of the cells. To assess this we measured the activity of the mitochondrial marker enzyme citrate synthase (CS) after stimulation with high glucose or TGF- β. A short exposure of podocytes for 48h with high glucose (30mM) did not induce changes in CS activity (Figure 3A, panel a). In contrast, prolonged exposure to high glucose increased the activity of the enzyme compared to the osmotic controls by 42 ± 13 % (p = 0.002, Figure 3A, panel b). When we stimulated podocytes with TGF-β (5ng/ml) for 24 h, we observed a significant decrease in the activity of CS. (87 ± 2 % of control mean, p = 0.043 Figure 3A, panel c). These data indicate that both factors can modulate the mitochondrial activity in podocytes.


a
n.s.
nmol/min/mg protein
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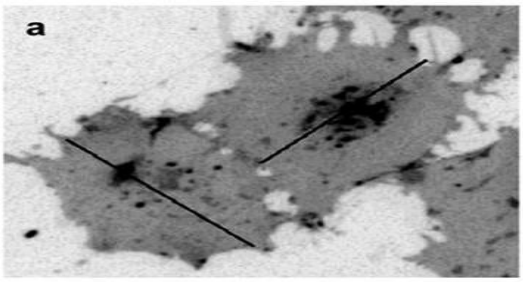
control
TGF-β
control
TGF-β

Figure 3A: Influence of glucose and TGF- β on citratsynthase activity in podocytes. Measurement of citratsynthase activity in differentiated podocytes after stimulation with 30mM glucose and 30mM mannitol as osmotic control for 48 h (a), 10 passages (b) or TGF-β (5ng/ml) (c) for 24 h, respectively. After incubation with oxalate and acetetyl-CoA citratsynthase activity was measured spectrophotometrically at a wavelength of 232nm. Each experiment was normalized to the total amount of protein. (Results are given as means ± S.D. (n=3); *p < 0.05 compared by Student's t-test; n.s. not significant).

5.7. Analysis of cell size

Prolonged exposure to high glucose leads to podocyte hypertrophy and changes in the mitochondrial content. When we analyzed the influence of glucose stimulation and TGF- β exposure on podocyte cell size, we found no difference in cell size of podocytes stimulated for 48h with 30mM glucose compared to 30mM mannitol, however under both conditions we detected a small but significant decrease in podocyte diameter, presumably as a result of the osmotic change of the milieu (Figure 3B, panel b). In contrast, cells cultured for 10 passages in 30mM glucose were significantly larger compared to the osmotic controls (p=0.003) (Figure 3B, panel c).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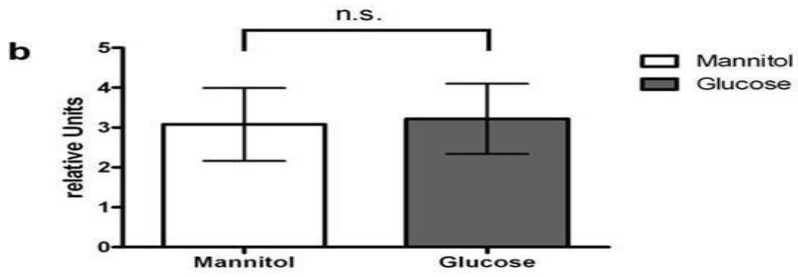
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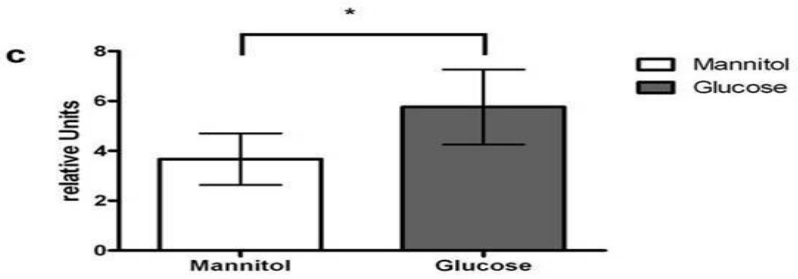
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Glucose
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Glucose

Figure 3 B: Prolonged exposure to high glucose leads to podocyte hypertrophy. Measurement of diameter of podocytes stained with choleratoxin (a) after treatment with 30mM glucose/30mM mannitol for 48h (b) or 10 passages (c). For each condition 50 cells were measured and the average was calculated in relative units. (n.s. not significant. *p< 0.05 compared by Student's t-test).

5.8. Analysis of mitochondrial marker

Mitochondrial marker expression is increased in podocytes after prolonged exposure to high glucose. To visualize the mitochondria content of living cells we used Mitotracker Orange™ to selectively stain only mitochondria with intact membrane potential. Therefore our staining reveals not only mitochondrial content and cytoplasmic distribution but indicates also their functional integrity (in Figure 3C, panel a representative image of control cells). Incubation of podocytes with 30mM glucose for 48h had no effect on the fluorescence signal of the mitochondrial marker, whereas in podocytes cultured for 10 passages in 30mM glucose we found a significantly higher fluorescence signal (p=0.011) compared to the 30mM mannitol controls. We also wanted to analyze changes in mitochondrial distribution. We could not detect changes in podocytes stimulated with 30mM glucose/30mM mannitol after 48h or 10 passages (data not shown). In both groups we detected a slight perinuclear accumulation of Mitotracker Orange™ and some peripheral staining in the cellular processes (as depicted in Figure 3C, panel a). These data indicate that prolonged exposure to glucose can induce podocyte hypertrophy and can lead to higher mitochondrial content of podocytes.

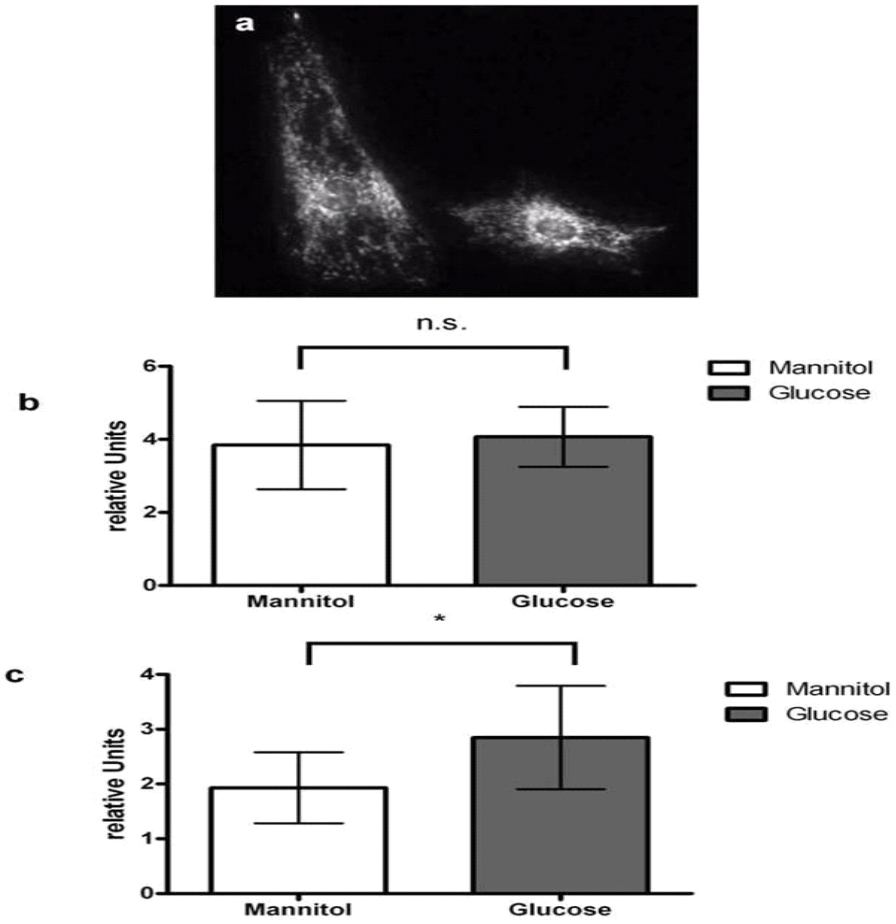
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Figure 3 C: Mitochondrial marker expression is increased in podocytes after prolonged exposure to high glucose. Measurement of Mitotracker OrangeTM in stained podocytes. (a) representative image and fluorimetric quantification in podocytes stimulated with 30mM glucose/30mM mannitol for 48h (b) or 10 passage (c). Scale bares represent the average of 12 wells for each condition and are expressed in relative units (*p < 0.05 compared by Student's t-test; n.s, not significant).

5.9. NADH-turnover

Glucose and TGF-β enhance NADH-turnover. To further characterize the general metabolic state of the cells we measured NADH-turnover in podocytes. The NADH-turnover was measured as time-dependent reduction of resazurin (alamar blue) in podocytes (Figure 4). The level of reduction of resazurin to resofurin as a marker for the amount of NADH in the cell was significantly higher in podocytes exposed to 30mM glucose compared to the mannitol controls after 48h at all measured time points (p=0.003 at 1h; p= 0.003 at 2h and p=0.001 at 3h; Figure 4, panel a). Culturing podocytes under 30mM glucose for 10 passages potentiated this effect at every time point (p <0.001 for all time points, Figure 4, panel a) in comparison to the osmotic control. Interestingly, podocytes stimulated with 5ng/ml TGF-β also showed a stronger reduction of the dye compared to the control 1h (p=0.015), 2h (p= 0.023) and 3h (p=0.002) after addition of resazurin (Figure 4, panel c). These data indicate a higher metabolic activity of podocytes in the presence of high glucose and TGF- β.

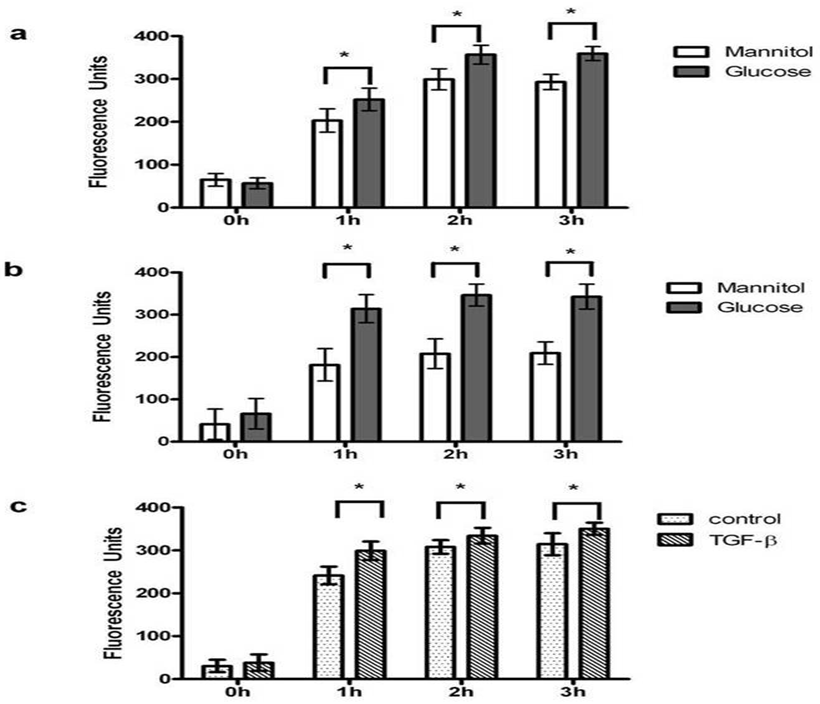

Figure 4: High glucose and TGF-β increase NADH-turnover in podocytes. Fluorometric measurement of reduction of resazurin (alamar blue) to resofurin in podocytes stimulated with 30mM glucose / 30mM mannitol for 48 h (a) or for 10 passages (b) or with 5ng/ml TGF-β for 24h (c) at 0h, 1h, 2h and 3h after addition of the alamar blue dye. (Results are given as mean ± S.D. *p <0.05 compared by Student`s t-test).

6. Discussion

Podocytes are very susceptible to several cellular stress factors. In diabetes, the podocyte has to adapt to a hyperglycemic milieu with acute blood sugar peaks as well as constantly elevated glucose levels. We have previously shown that in the initial phase in type I as well as in type II diabetes in mice, podocytes get lost during the development of diabetic glomerulosclerosis [1]. However, after an initial loss podocyte numbers remain stable despite ongoing elevation of blood glucose levels. This indicates that podocytes are able to adapt to hyperglycemic stress. TGF-β activation is a well described event in the course of diabetic nephropathy [20,21,22,23,24,25]. High concentrations of active TGF-β as detected in diabetic kidneys induce pro-apoptotic signaling cascades in podocytes including p38MAPK and pro-apoptotic SMAD signaling pathways, whereas low levels have a more pro-differentiating effect and enhance expression of differentiation markers such as synaptopodin [26]. In this manuscript we presented for the first time high glucose and TGF-β induced effects on the OCRs in podocytes. Podocytes cultured for 10 passages in 30mM glucose showed an increased OCR indicating an elevated ATP-demand in these cells. OCRs after oligomycin injection represents the proton leak in a cell. As a consequence of elevated NADH levels, the electromotive force raises and drives the chronic podocytes into an increased dissipation as we observed in our profiles, indicating a major proton leak in these cells independent of ATP-production. The OCR increase after FCCP injection is related to an enhanced respiratory capacity. The OCR measurements with the Seahorse XF24 extracellular flux analyzer represent

the summation of all oxygen consuming processes in the cell like mitochondrial respiration through the OXPHOS, glycolysis or production of reactive oxygen species (ROS), so probably not only one mechanism alone is responsible for changed OCRs in these cells. Interestingly, extracellular acidification rates (ECAR) which represent the glycolytic component in cells, was unaffected consistent with the observation of *Abe and coworkers* [9,20]. They postulated that podocytes produce energy mainly by oxidative phosphorylation whereas glycolysis is not essential for these cells. They also suggested that podocytes are not able to adapt their energy metabolism to changing environmental conditions. However our results clearly indicate that podocytes are able to enhance OCR in response to a changed environment. One possibility for the cell to raise oxygen consumption is to accelerate mitochondriogenesis. To prove this hypothesis we performed fluometrical analysis of the mitochondrial marker Mitotracker Orange™ under diabetic conditions and observed an increased signal in the chronic diabetic podocytes which indicates elevated mitochondria content. In parallel we detected cellular hypertrophy, as indicated by a significant increase in cell size. When we analyzed citrate synthase activity under the above described conditions *in vitro*, we found an enhanced activity of the enzyme in the cells when cultured in 30mM high glucose for 10 passages which could be related to increased levels of respiratory chain proteins. In contrast, TGF-β stimulated cells showed a reduced citrate synthase activity which indicates that TGF-β modulates the protein levels negatively or influences indirect the protein catabolism. Surprisingly, high glucose exposure for a short time has no effect

on the activity of the enzyme. This would indicate that only sustained hyperglycemia leads to an upregulation of respiratory chain proteins, whereas the stimulation with a pro-apoptotic concentration of TGF-β leads to down-regulation of citrate synthase or OXPHOS protein levels, respectively. The elevated activity of citrate synthase as discussed above correlates well with an increased amount of mitochondrial respiratory chain proteins under prolonged glucose exposure. Even though we detected a similar increase in OCRs in podocytes stimulated with TGF-β, we detected a decrease in citrate synthase activity corresponding with lower OXPHOS protein levels. A possible explanation to achieve this elevated aerobic condition is a change of mitochondrial structure to bring more mitochondria into an active state. This is also well in line with our fluometrical results since the Mitotracker Orange™ assay measures only mitochondria with an active mitochondrial potential. Surprisingly, the same effect on OCRs as described for the podocytes after prolonged glucose exposure was observed in podocytes stimulated with TGF-β for 24h. In this context it is important to discuss that reactive oxygen species (ROS) can cause elevated OCRs as postulated in other tissues [27,28]. A significant generation of ROS in pro-apoptotic relevant concentrations has been described in podocytes after short time exposure to high glucose and TGF-β [3,29,30,31]. However, we did not find an elevated OCR in podocytes exposed to high glucose for 48h (Figure 2). Moreover, we did not find significantly increased ROS levels in podocytes after prolonged glucose exposure (data not shown). This would indicate that these cells are protected via anti-oxidative mechanisms and that the elevated oxygen utilization that we

detected in our experiments is ROS independent. It is well known that respiration uncoupling proteins are upregulated in diabetes [32]. *Hong et al.* demonstrated in insulinoma cells that overexpression of the uncoupling protein 2 (UCP2) leads to an increased mitochondrial respiration with an elevated state 4 indicating an increased proton leak or uncoupled respiration [33]. Furthermore, *Friederich et al.* could show that UCP2-expression is changed in diabetic cells and correlated with an increased oxygen consumption [34]. We observed increased OCRs in podocytes after prolonged glucose exposure and in podocytes stimulated with TGF-β after oligomycin injection indicating a higher proton leak. Even though an increased proton leak could be a sign of a damage within the cell, upregulation of UCPs, which control both mitochondrial ROS-production and apoptosis, can also be protective for the cell [35]. We suggest that this pathway is a possible explanation for the changed proton leak in podocytes prolonged exposed to high glucose and under influence of TGF-β. This could be interpreted as a way of the cells to protect themselves from oxidative stress and cell death. In addition, we also found an increase in non-mitochondrial OCRs in the podocytes after prolonged glucose exposure as well as in podocytes stimulated with TGF-β indicating an elevated activity of various desaturase and detoxification enzymes as well as an increased activity of non-mitochondrial NADPH oxidases which could be responsible for the elevated cellular oxygen uptake. *Edlund et al*. recently showed an influence of NADPH oxidases on the increased oxygen consumption in diabetic proximal tubule cells and demonstrated that increased oxidative stress is responsible for the

elevated oxygen utilization of the diabetic renal tubule cells [36]. A recent study demonstrated that human mesangial cells are able to develop opposing mechanisms against glycooxidative stress [37]. Our data support a similar mechanism in podocytes. Interestingly, we found that glycolytic activity as expressed by extracellular acidification rates (ECARs) are not affected. It is evident that factors that play a major role in diabetic nephropathy lead to hypertrophy of podocytes *in vivo[23,38,39]*. Even though this has been documented *in vivo*, we are the first to demonstrate a change of cell size in podocytes after prolonged exposure to high glucose *in vitro.* We demonstrated with our result that a high glucose milieu alone is able to cause an increase in cell size. In a study by *Petermann and coworkers,* hypertrophy in podocytes was induces by mechanical stress *in vitro* as a consequence of cell cycle inhibition and elevation of $p27^{kip}$ [40]. This in itself could also account for many adaptive metabolic changes that we detected since a difference in cell size would require changes in cellular energy supply. The OCR measurements with the Seahorse Extracellular Flux Analyzer reflect all oxygen consuming processes in living cells that are influenced by the interaction of all pathways relevant for the ATP-production. In contrast, the oxidative phosphorylation (OXPHOS) assays focus on a single enzyme by interrupting the electron transfer and exclude the influence of the other complexes. In diabetes, high glucose–induced changes in highly aerobic tissues such as heart, brain, skeletal muscle and kidney are mainly found in complex I, complex III and complex IV, whereas ATPase as complex V can also be affected [41,42,43,44]. However, mitochondrial function in podocytes

was not systematically analyzed so far. Although we expected an increase in respiratory chain complexes in the chronic podocytes due to the measured oxygen consumption rates we only observed an elevated activity of NADH-dehydrogenase (complex I) and cytochrome c oxidase (complex IV) under acute high glucose conditions. This could be explained with the increased NADH availability as we detected by alamar blue assay and an increased need of oxygen as a consequence of the elevated production of reactive oxygen species as described in other studies. The decrease of ATPase activity might be a result of elevated levels of NADH to protect the cells from an overproduction of ATP and finally inhibition of the respiratory chain complexes. Interestingly, we detected similar results in the respiratory chain activities in podocytes after prolonged glucose exposure and in the cells treated with TGF-β with a more pronounced effect in podocytes after glucose exposure. They displayed a decreased activity of NADH- dehydrogenase (complex I) and ATPase (complex V) and a slight but not significant upregulation of succinate dehydrogenase and ubiquinone cytochrome c reductase (complex II + III). A downregulation of ATPase's activity due to uncoupled respiratory chain is described previously [45]. So this effect detected under both conditions is consistent with the increased proton leak of these cells. A previous study postulated a relationship between the activity of cytochrome c oxidase and production of reactive oxygen species in lung epithelial cells [46]. So we suggest that the significant decrease in cytochrome c oxidase activity observed in podocytes treated with a pro-apoptotic concentration of TGF-β is likely related to an increased production of ROS.

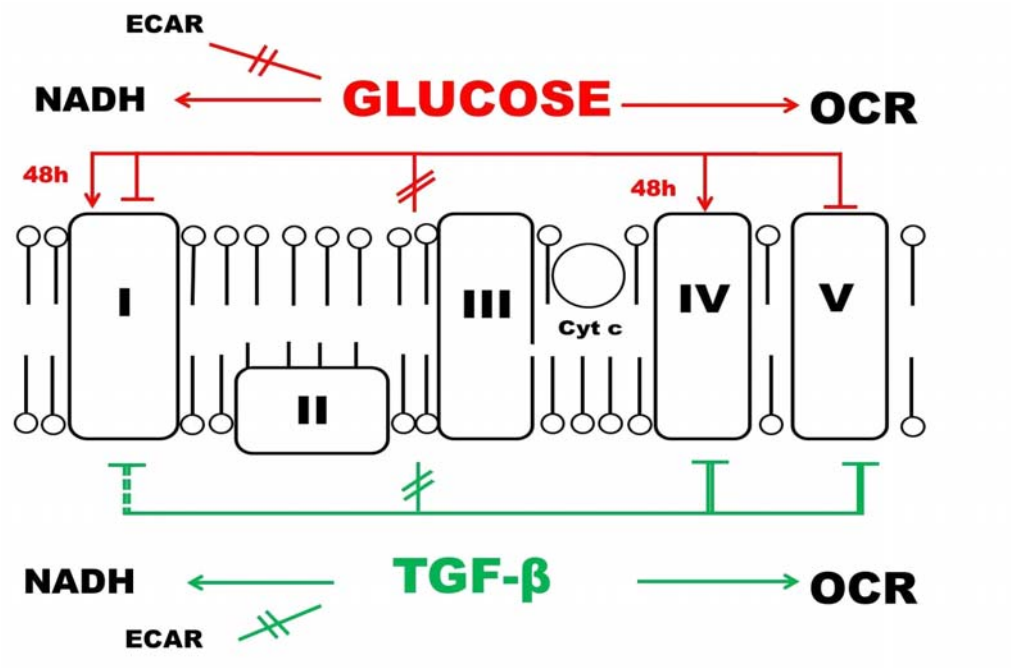

Figure 5: Schematic overview of Glucose and TGF-b influences on respiratory chain complex activity in podocytes

In contrast, the elevated OCRs after TGF-β stimulation are probably not linked to citrate synthase activity or lower protein levels. It is well described, that

increased NADH concentrations in diabetes induce mitochondrial dysfunction [4,47]. Using resazurin as an indicator of cytosolic NADH concentrations, we found an increased reduction to resofurin in all tested conditions consistent with increased glucose metabolism. In this study we detected this effect already in podocytes stimulated with high glucose for 48h and with TGF-β and this effect is strongly amplified in podocytes after prolonged culture in high glucose. So we postulate that the changed NADH-turnover is an early event in the development of diabetic podocytopathy. Furthermore, these results may indicate changes in the polyol pathway which has been not described in diabetic podocytes, but plays an important role in other diabetic cells and tissues[48,49,50,51,52,53]. In the polyol pathway, glucose is converted to sorbitol and finally fructose by using NADPH and increasing NADH levels. If the increased polyol pathway leads to an activation of mitochondrial oxygen species or vice versa is controversially discussed in the literature[4,47]. But it is clear that these two factors are connected to each other and may together lead to mitochondrial disturbance in the cell. If the metabolic rates raise and thus the ROS production, expression of uncoupling proteins 2 (UCP-2) is upregulated and lowers the mitochondrial membrane potential leading to a decreased ATP synthesis. So in summary high glucose exposure and TGF-β influence podocytes metabolism. The observed changes may drive the podocytes towards mitochondrial disturbance and the metabolic effects may sensitize the cells towards apoptosis.

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Hannover, 18.07.2011

Danksagung

An dieser Stelle möchte ich mich bei allen ganz herzlich bedanken, die zum Gelingen meiner Doktorarbeit beigetragen haben.

Herr Prof. Dr. med. Mario Schiffer für die Überlassung des interessanten Themas , die Betreuung und Leitung dieser Arbeit , sowie die freundliche Unterstützung bei der Erstellung dieser Arbeit.

Frau Dr. rer.nat. Kirstin Worthmann für die kompetente Einarbeitung in die Thematik und Unterstützung bei der Durchführung der Experimente sowie die stetige Bereitschaft, mich bei auftretenden Fragen und Problemen fachlich zu beraten.

Allen Labormitgliedern der Arbeitsgruppe Prof. M. Schiffer, Klinik für Nieren- und Hochdruckerkrankungen, Medizinische Hochschule Hannover, der Arbeitsgruppe Prof .A.M. Das, Abteilung für Pädiatrische Nieren-, Leber- und Stoffwechselerkrankungen, Medizinische Hochschule Hannover und der Arbeitsgruppe Dr. S.Engeli, Institut für klinische Pharmakologie, Medizinische Hochschule Hannover, für die freundliche Unterstützung im Laboralltag, sowie die Bereitschaft zu fachlichen Diskussionen .

Herr Prof. Dr. med. vet. Thomas Lutz, Veterinärphysiologisches Institut der Vetsuisse- Fakultät Zürich, für die freundliche Übernahme des Hauptreferates.

Der Gottfried und Julia Banger-Rhyner – Stiftung für die finanzielle Unterstützung durch ein zinsloses Darlehen.

Einen besonderen Dank an meine Mutter für die Ermöglichung dieser Ausbildung und immerwährende Unterstützung .